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<b>(54) Title:</b> FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF		
<b>(57) Abstract</b>  The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are methods of identifying nucleic acid sequence encoding the fluorescent proteins and further analyzing the proteins.		

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**FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES  
OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND  
USES THEREOF**

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**BACKGROUND OF THE INVENTION**

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, methods of identifying the DNA sequences encoding the proteins and  
15 uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for  
20 marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps,  
25 however, make the process laborious and difficult to control. An alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include  $\beta$ -galactosidase, firefly luciferase

and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

5 A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

10 Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing  
15 subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by  
20 Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets  
25 in a compact structure, where, in the center, an alpha helix containing the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in

general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

### SUMMARY OF THE INVENTION

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence  
5 encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is  
10 provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid  
15 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a  
20 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the  
25 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an

intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers).

Figure 2A shows multiple alignment of novel fluorescent proteins. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP). Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming beta-sheets are underlined; the residues whose side chains form the interior of the beta-can are shaded (according to Yang et al., *Nature Biotechnol.* 14, 1246-1251 (1996)). Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

Figure 3 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 4 shows the excitation and emission spectrum of the novel fluorescent protein from *Clavularia*, cFP484.

Figure 5 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP506.

5 Figure 6 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP538.

Figure 7 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma striata*, dsFP483.

10 Figure 8 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, drFP583.

Figure 9 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia sulcata*, asFP600.

Figure 10 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dgFP512.

15 Figure 11 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dmFP592.

## DETAILED DESCRIPTION OF THE INVENTION

20 As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

25 As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., *Nature* 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for



expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

15 A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

25 A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3'

(carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination  
5 sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

10 The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that  
15 provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining  
20 the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a  
25 transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by  
5 exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to  
10 eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter  
15 cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an  
20 identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example,  
25 heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

5           The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: 10 serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine; X: any residue). NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552- 15 59 is used.

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

20           In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group 25 consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a

fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid  
5 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a  
10 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the  
15 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an  
20 intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

**EXAMPLE 1**Biological Material

- 5                    Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

10

**TABLE 1**Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp.	Western Pacific	green spots on oral disk

"green"		
Anemonia sulcata	Mediterranean	purple tentacle tips

**EXAMPLE 2****cDNA Preparation**

Total RNA was isolated from the species of interest  
5 according to the protocol of Chomczynski and Sacchi (Chomczynski P.,  
et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was  
synthetized starting with 1-3 µg of total RNA using SMART PCR cDNA  
synthesis kit (CLONTECH) according to the provided protocol with the  
only alteration being that the "cDNA synthesis primer" provided in the  
10 kit was replaced by the primer TN3 (5'- CGCAGTCGACCG(T)<sub>13</sub>, SEQ ID  
No. 1) (Table 2). Amplified cDNA samples were then prepared as  
described in the protocol provided except the two primers used for PCR  
were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2)  
(Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration.  
15 Twenty to twenty-five PCR cycles were performed to amplify a cDNA  
sample. The amplified cDNA was diluted 20-fold in water and 1 µl of  
this dilution was used in subsequent procedures.



### Oligos Used in cDNA Synthesis and RACE

25

**EXAMPLE 3**Oligo Design

To isolate fragments of novel fluorescent protein cDNAs,  
5 PCR using degenerate primers was performed. Degenerate primers  
were designed to match the sequence of the mRNAs in regions that  
were predicted to be the most invariant in the family of fluorescent  
proteins. Four such stretches were chosen (Table 3) and variants of  
degenerate primers were designed. All such primers were directed to  
10 the 3'-end of mRNA. All oligos were gel-purified before use. Table 2  
shows the oligos used in cDNA synthesis and RACE.

**TABLE 3**

Key Amino Acid Stretches and Corresponding Degenerate Primers Used  
for Isolation of Fluorescent Proteins

5

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5)  GEGNG (SEQ ID No. 8)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7) GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

**EXAMPLE 4**Isolation of 3'-cDNA Fragments of nEPs

The modified strategy of 3'-RACE was used to isolate the target fragments (see Figure 1). The RACE strategy involved two consecutive PCR steps. The first PCR step involved a first degenerate primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3' portion identical to the TN3 primer used for cDNA synthesis (for sequence of T7-TN3, Table 2). The reason for substituting the longer T7-TN3 primer in this PCR step was that background amplification which occurred when using the shorter TN3 primer was suppressed effectively, particularly when the T7-TN3 primer was used at a low concentration (0.1  $\mu$ M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-9002). The second PCR step involved the TN3 primer (SEQ ID No. 1, Table 2) and a second degenerate primer (Table 4).

**TABLE 4**

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

5

The first PCR reaction was performed as follows: 1 µl of 20-fold  
 10 dilution of the amplified cDNA sample was added into the reaction  
 mixture containing 1X Advantage KlenTaq Polymerase Mix with  
 provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate

primer (Table 4) and 0.1  $\mu$ M of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20  $\mu$ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1  $\mu$ l of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200  $\mu$ M dNTPs, 0.3  $\mu$ M of the second degenerate primer (Table 4) and 0.1  $\mu$ M of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

**EXAMPLE 5**Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel  
5 fluorescent protein cDNAs, two nested 5'-directed primers were  
synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were  
then amplified using two consecutive PCRs. In the next PCR reaction,  
the novel approach of "step-out PCR" was used to suppress background  
amplification. The step-out reaction mixture contained 1x Advantage  
10 KlenTaq Polymerase Mix using buffer provided by the manufacturer  
(CLONTECH), 200  $\mu$ M dNTPs, 0.2  $\mu$ M of the first gene-specific primer  
(see Table 5), 0.02  $\mu$ M of the T7-TS primer (SEQ ID No. 18), 0.1  $\mu$ M of  
T7 primer (SEQ ID No. 19) and 1  $\mu$ l of the 20-fold dilution of the  
amplified cDNA sample in a total volume of 20  $\mu$ l. The cycling profile  
15 was (Hybaid OmniGene Thermocycler, tube control mode): 23-27  
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of  
amplification was diluted 50-fold in water and one  $\mu$ l of this dilution  
was added to the second (nested) PCR. The reaction contained 1X  
Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH),  
20 200  $\mu$ M dNTPs, 0.2  $\mu$ M of the second gene-specific primer and 0.1  $\mu$ M  
of TS primer (SEQ ID No. 2) in a total volume of 20  $\mu$ l. The cycling  
profile was (Hybaid OmniGene Thermocycler, tube control mode): 12  
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of  
amplification was then cloned into pAtlas vector (CLONTECH) according  
25 to the manufacturer's protocol.

**TABLE 5**Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'- GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)



## EXAMPLE 6

### Expression of nFPs in *E. coli*

5 To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table  
10 6). Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and nFP. The PCR was performed as follows: 1  $\mu$ l of  
15 the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200  $\mu$ M dNTPs, 0.2  $\mu$ M of upstream primer and 0.2  $\mu$ M of downstream primer, in a final total volume of 20  $\mu$ l. The cycling profile was (Hybaid OmniGene  
20 Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard  
25 protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium (supplemented with 100  $\mu$ g/ml of ampicillin) at 37°C overnight. 100  $\mu$ l

of the overnight culture was transferred into 200 ml of fresh LB medium containing 100  $\mu\text{g/ml}$  of ampicillin and grown at 37°C, 200 rpm up to  $\text{OD}_{600}$  0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The  
5 cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

**TABLE 6**

Primers Used to Obtain Full Coding Region of nFPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctctttcaaca agttatc (SEQ ID No. 36) BamHI	5'-tagtactcgagcttattcgta tttcagtgaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacattttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcaaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgagggttgaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttatc (SEQ ID No. 43) BamHI	5'-tagtactcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttccttttaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgtccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgaggccattacg ctaac (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtgcaactaaagaagaatg (SEQ ID No. 51)	5'-tagtactcgagattcggtttaat gccttg (SEQ ID No. 52)

**EXAMPLE 7****Novel Fluorescent Proteins and cDNAs Encoding the Proteins**

Seven cDNA full-length cDNAs encoding fluorescent  
5 proteins were obtained (SEQ ID Nos. 45-51), and seven novel  
fluorescent proteins were produced (SEQ ID Nos. 53-59). The spectral  
properties of the isolated novel fluorescent proteins are shown in Table  
7, and the emission and excitation spectra for the novel proteins are  
shown in Figures 3-11.

10

**TABLE 7**Spectral Properties of the Isolated NFPs.

Species	NFP Name	Abs. Max. n m	Emission Maximum n m	Maximum Extinction Coeff.	Relative Quantum Yield*	Relative Brightness **
Anemonia majano	amFP486	458	486	40,000	0.3	0.43
Clavularia sp.	cFP484	456	484	35,300	0.6	0.77
Zoanthus sp.	zFP506	496	506	35,600	0.79	1.02
Zoanthus sp.	zFP538	528	538	20,200	0.52	0.38
Discosoma sp. "red"	drFP583	558	583	22,500	0.29	0.24
Discosoma striata	dsFP483	443	483	23,900	0.57	0.50
Anemonia sulcata	asFP600	572	596	56,200	<0.001	-
Discosoma sp "green"	dgFP512	502	512	20,360	0.3	0.21
Discosoma sp. "magenta"	dmFP592	573	593	21,800	0.11	0.09

5 \*relative quantum yield was determined as compared to the quantum yield of *A. victoria* GFP.

\*\*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

Multiple alignment of fluorescent proteins is shown in Figure 2A. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP, SEQ ID No. 54). The amino acid sequences of the novel fluorescent proteins are labeled as SEQ ID Nos. 55-63. Two  
5 proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming  $\beta$ -sheets are underlined; the residues whose side  
10 chains form the interior of the  $\beta$ -can are shaded. Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

The following references were cited herein.

1. Ormo et al., (1996) Science 273: 1392-1395.
- 15 2. Yang, F., et al., (1996) Nature Biotech 14: 1246-1251.
3. Cormack, et al., (1996) Gene 173, 33-38.
4. Haas, et al., (1996) Current Biology 6, 315-324.
5. Yang, et al., (1996) Nucleic Acids Research 24, 4592-4593.
6. Ghoda, et al., (1990) J. Biol. Chem. 265: 11823-11826.
- 20 7. Prasher D.C. et al. (1992) Gene 111:229-33.
8. Kain et al. (1995) Biotechniques 19(4):650-55.
9. Chomczynski P., et al., (1987) Anal. Biochem. 162, 156-159.
10. Frohman et al., (1998) PNAS USA, 85, 8998-9002.

Any patents or publications mentioned in this specification  
25 are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, 5 molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope 10 of the claims.

**WHAT IS CLAIMED IS:**

1. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

5 screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

10

2. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence hybridizes to a primer  
15 selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

3. A method of analyzing a fluorescent protein in a cell,  
20 comprising the steps of:

a) expressing a nucleic acid sequence encoding a fluorescent protein in said cell, wherein said protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63;  
and

25 b) measuring a fluorescence signal from said protein.

4. The method of claim 3, further comprising the step of:

sorting said cell according to said signal.



5. The method of claim 4, wherein said step of sorting comprises sorting said cell by fluorescence activated cell sorting.

5           6. The method of claim 3, wherein said nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to said fluorescent protein, wherein said protein of interest is distinct from said fluorescent protein.

10           7. The method of claim 6, wherein the fluorescence signal indicates a presence of said gene of interest in said cell.

8. The method of claim 7, wherein said cell further comprises a protein of interest fused to said fluorescent protein.

15

9. The method of claim 8, further comprising the step of:

identifying an intracellular location of said fluorescent protein, thereby identifying an intracellular location of said protein of  
20 interest.

10. An isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

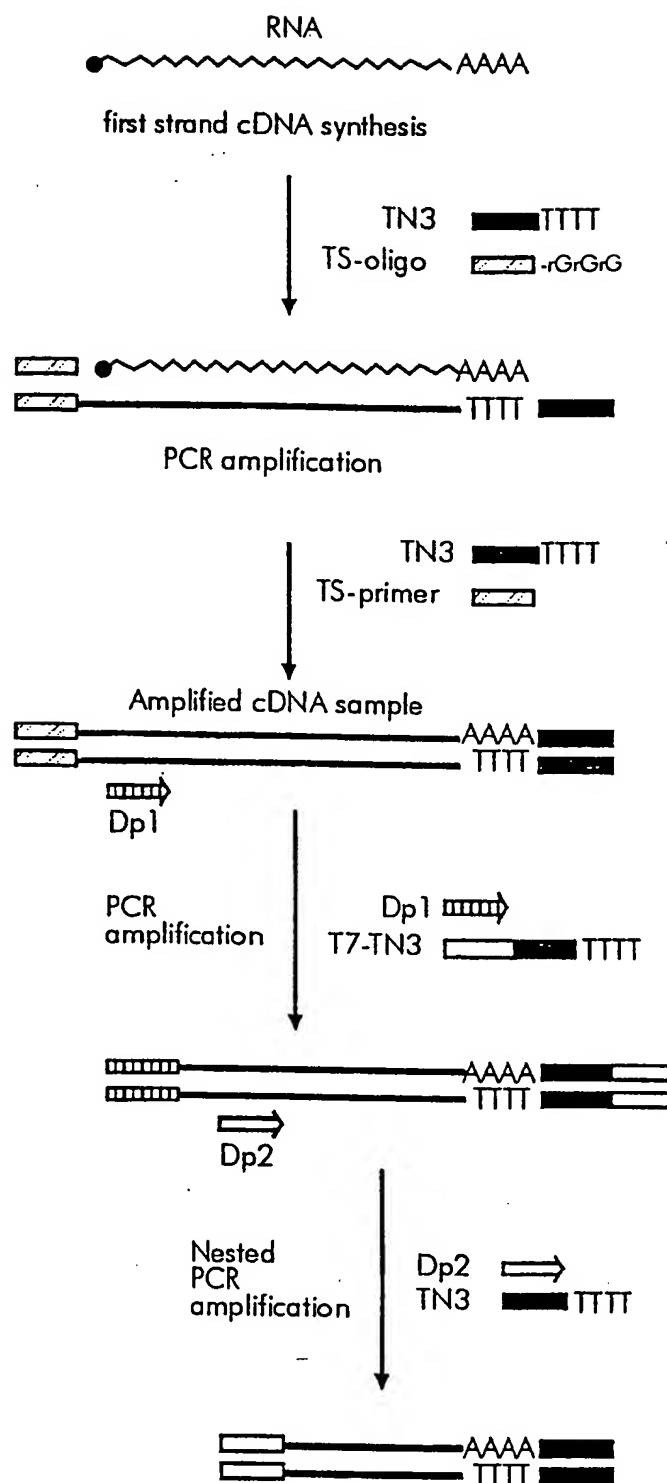


FIG. 1

1/11

SYNOPSIS OF THE INVENTION

10	20	30	40	50	SEQ ID#
MSKGEELFTG.VVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTT.GKLPVP..W GFP					54
MAQSKHGLTK.EMTMKYRMEGCVDGHKFVITGEGIGYPFKGKQAINLCVV..EGGPLPFAE					zFP506 57
--H-----KE.-----H-----N-----T-----I.-----S-					zFP538 58
MSWSKSVIKE.EMLIDLHLEGTfNGHYFEIKGKGKGNPNEGNTVTLEVT..KGGPLPFGW					dsFP483 59
...M-AL---Y-K-N-TM--VV--LP-K-R-D-----YQ-SQEL--T-V.-----SY					dgFP512 62
-RS--N-----F-REFKVRM--V---E---E-E-E-R-Y--H---K-K--.-----A-					drFP583 60
M-C--N-----F-REFKVRM--V---E-----E-E-R-Y--HCS-K-M--.-----AF					dmFP592 63
...MASFLKK.TMPFKTTIEGTVNGHYFKCTGKGEKNPFEGTQEMKIEVI..EGGPLPFAF					asFP600 61
MALSNKFIDG.DMKMTYIIMDGCNVNGHYFTVKGEGNGKPYEGTQTSTFKVTMANGGPLAFSE					amFP486 55
KALTTMGVIKPDMKIKLMMEGNVNGHAFVIEGEGEGKPYDGTHTLNLEVKMAEGAPLPFSY					cFP484 56
60	70	80	90	100	110
PTLVTTFSYGVQCFSTRYPDHMKQHDFKFSAM...PEGYVOERTIFFKDDGNYKTRAEVKFEGD.. GFP					
DILSAAFNYGNRVFTEYPQDIV..DYFKNSC...PAGYTWDRSFLFEDGAVCICNADITVSVEEN					
-----G-K--D-I-----G-----V-----K--					
HILCPQFQYGNKAFVHHPDDIP..DYLKLSF...PEGYTWRSMHFEDGGLCCITNDISLTGN..					
D--TTM-----R---NY-E---..-IF-QTCSGPNG--S-Q-T-TY---V-TA-SN--VV-D..					
D--S-----S-VY-K--A---..-K-----FK--V-N-----VVTV-Q-S--QDG..					
D--S-----S-VY-K--A---..-K-----FK--V-N-----VVTVSQ-S--KDG..					
HILSTSCMYGSKTFIKYVSGIP..DYFKQSF...PEGFTWERTTTTYEDGGFLTAHQDTSLDGD..					
DILSTVFKYGNRCFTAYPTSMF..DYFKQAF...PDGMSYERTFTYEDGGVATASWEISLKN..					
DILSNAFQYGNRALTKYPDDIA..DYFKQSF...PEGYSWERTMTFEDKGIVKVKSDISMEED..					
120	130	140	150	160	170
TLVNRIELKGIDFKEDGNILGHKLEYNYNHNVYIMADKQKNGIKVNFKIRHNIEDGSVQL					
CMYHESKFYGVNFPADGPVM.KKMTDNWEPSCFKIIPVPKQGIILKGDVSMYI.LLKDGGRRLR					
-I--K-I-N-M-----T---A-----M-----Y-					
CENYDIKFTGLNFPPNGPVV.QKKTGTWEPSTERLYP..RDGVLIGDIHHAHALTEGGGHYV					
T-----H-M-A---LD--MM.--R-MK-----IMFE ---L-R-D-AMS-LLK-----R					
--I-KV--I-V---SD---M-----M---A-----K-E--K--KLKD---L					
--I-EV--I-V---SD---M--RR-R-----S-----K---M--RL-----L					
CLVYKVKILGNNFPADGPVM.QNKAGRWEPEATEIVYE..VDGVLRGQSLMALKCPGGRHLT					
CFEHKSTFHGVNFPADGPVM.AKKTGTWDPSFEKMTV..CDGILKGDVTAFLMLQGGGNYR					
SFIYEIRFDGMNFPNGPVV.QKKTWKWEPSTEIMYV..RDGVLVGDISHSLLEGGGHYR					
180	190	200	210	220	230
ADHYQQNTPIGDG.PVLLPDNHYLSTQSALSCKDPNEKRDHMLLEFVTAAGITHGMDELYK					
COFDTVYKAKSV..PRKMPDWHFIQHKLTREDRSDAKNQKWLTEHAIASGSALP					
-----S---E-----L-----Q-----FP---A					
CDIKTVYPAKK...PVKMPGYHYVDTKLVIRSNDKEFM.KVEEHEIAVARHHPQSQ					
--FE-I-KPN- V-----D--F--HYIE-T-QQNYN V--LT-V-E--YSS-EKIGKSKA					
VEF-SI-M---...-QL---Y---S--D-T-HNEDYT.I--QY-RTEG---LFL					
VEF-SI-MV-- PS-QL---Y---S--DMT-HNEDYT V--QY-KTQ-----FIKPLQ					
CHLHTTYRSKKPASALKMPGFHFEDHRIEIMEEVEKKGK.CYQYEAAGVGRYCDAAPSKLGHN					
CQFHTSYKTKK...PVTMPNHNHVEHRIARTDLKGGN.SVOLTEHAVAHITSVFPF					
CDFKSIYKAKK...VVKLPDYHFVDHRIEILNHDKDYN.KVTLYENAVARYSLPSQA					

FIG. 2A

»

MKCKFVFCLSFLVLAITNANI FLRNEADLEEKTLRIP

FIG. 2B

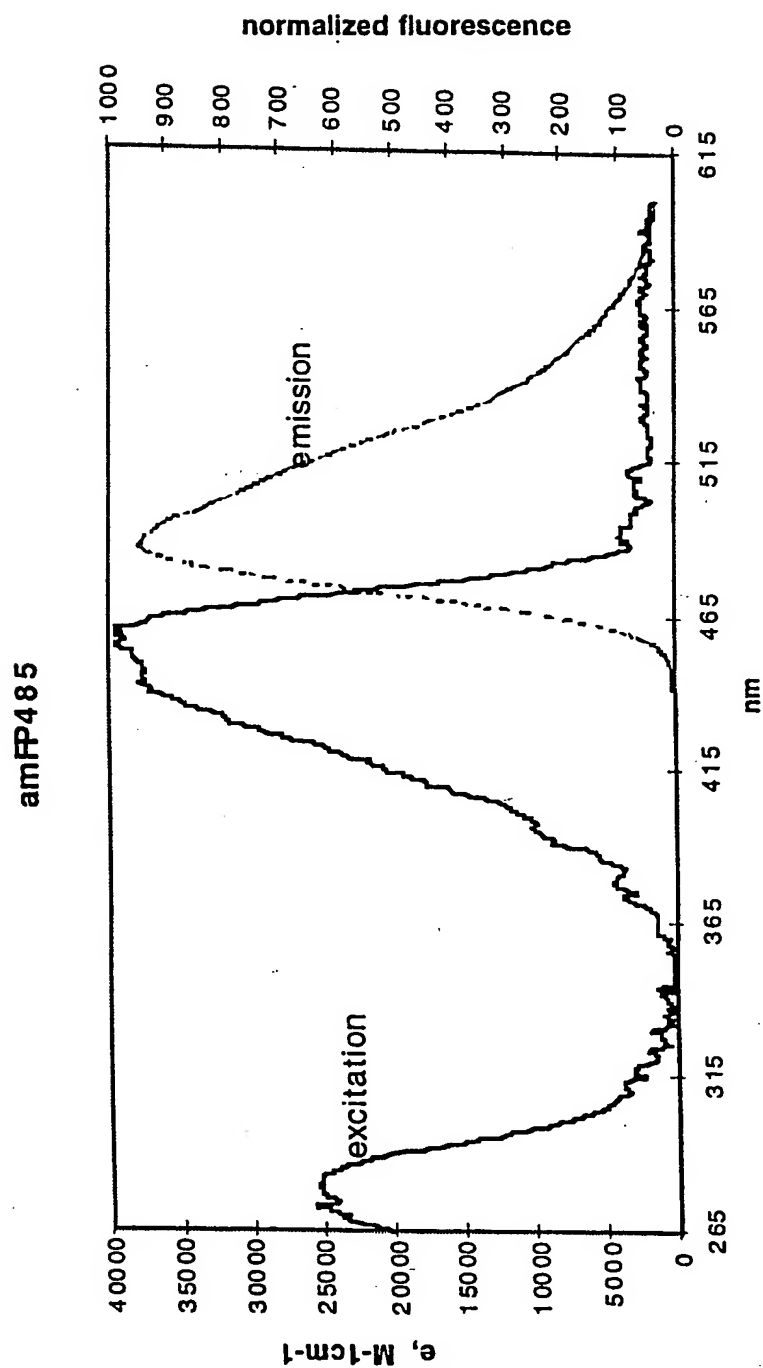


FIG. 3

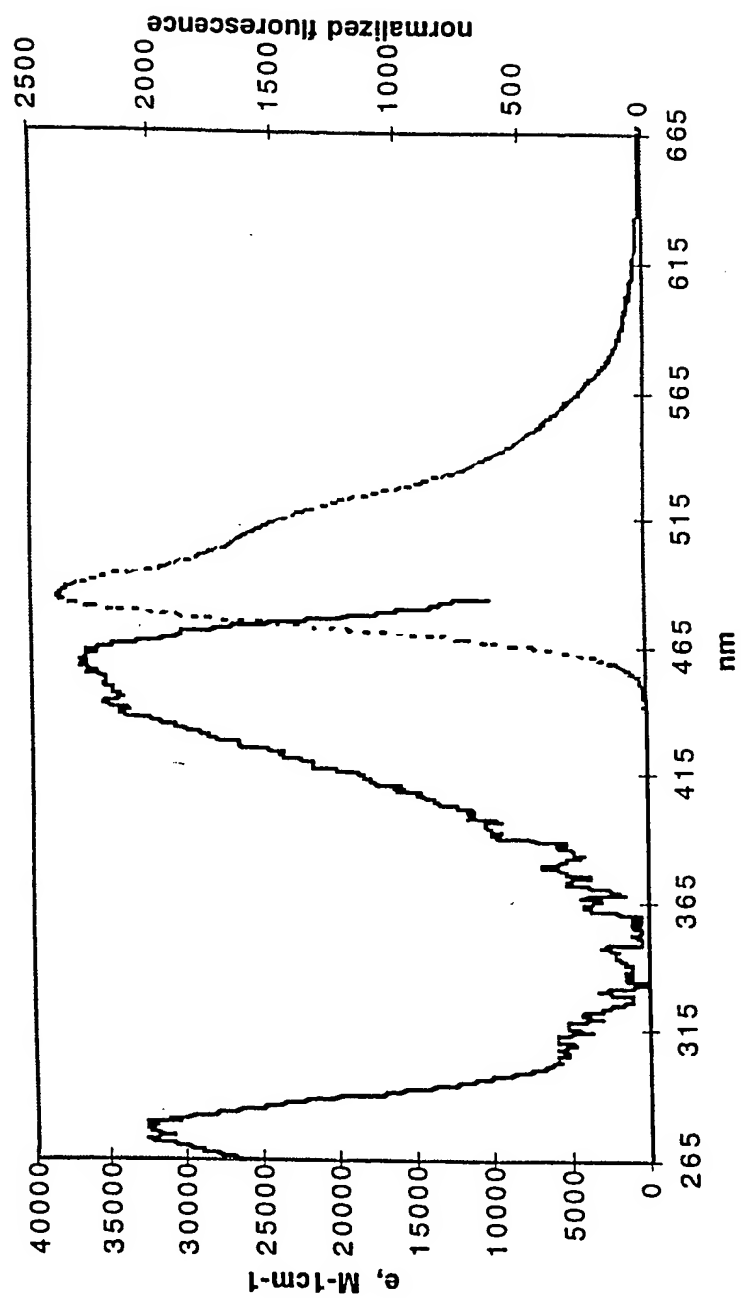


FIG. 4

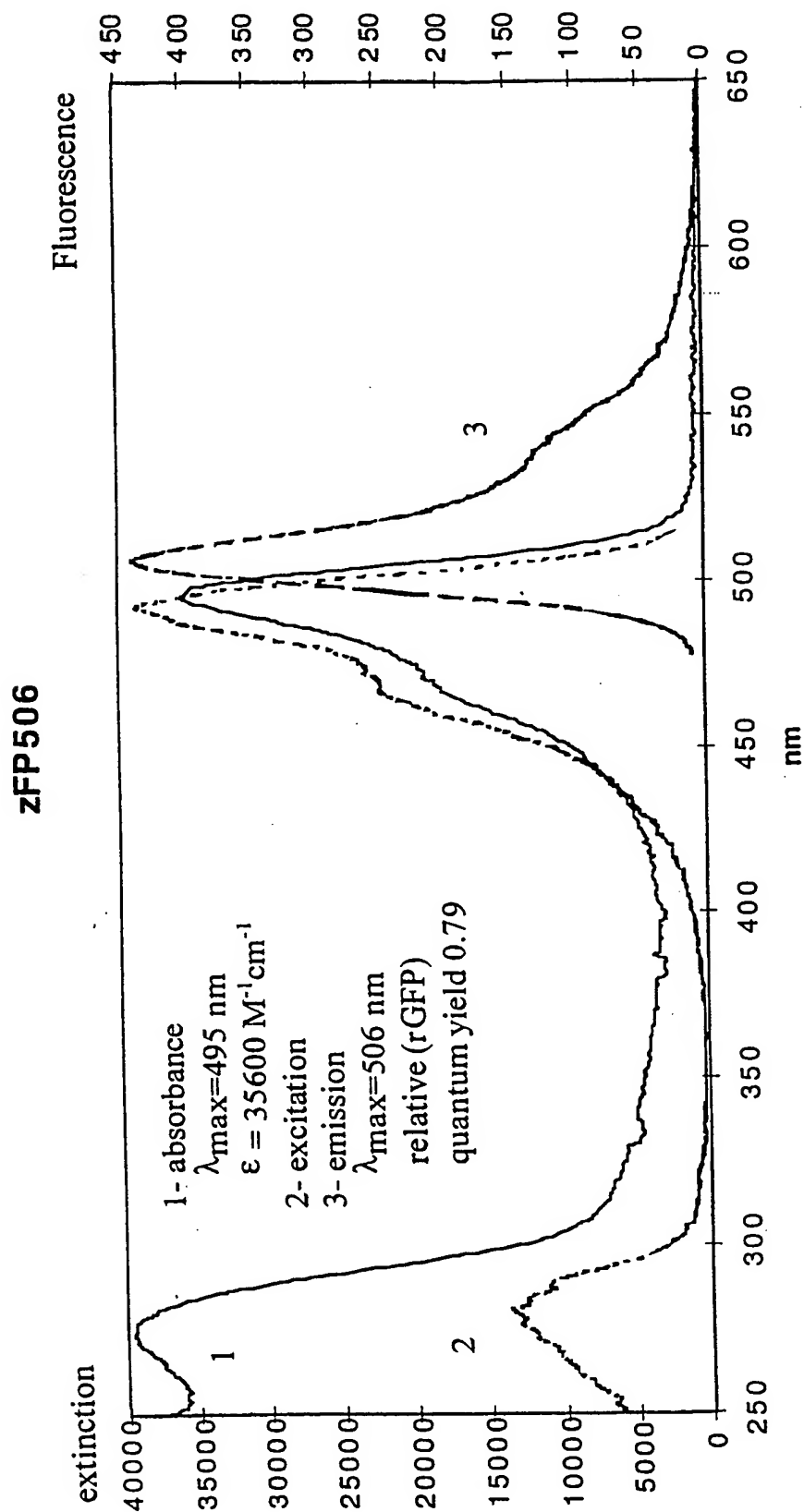


FIG. 5

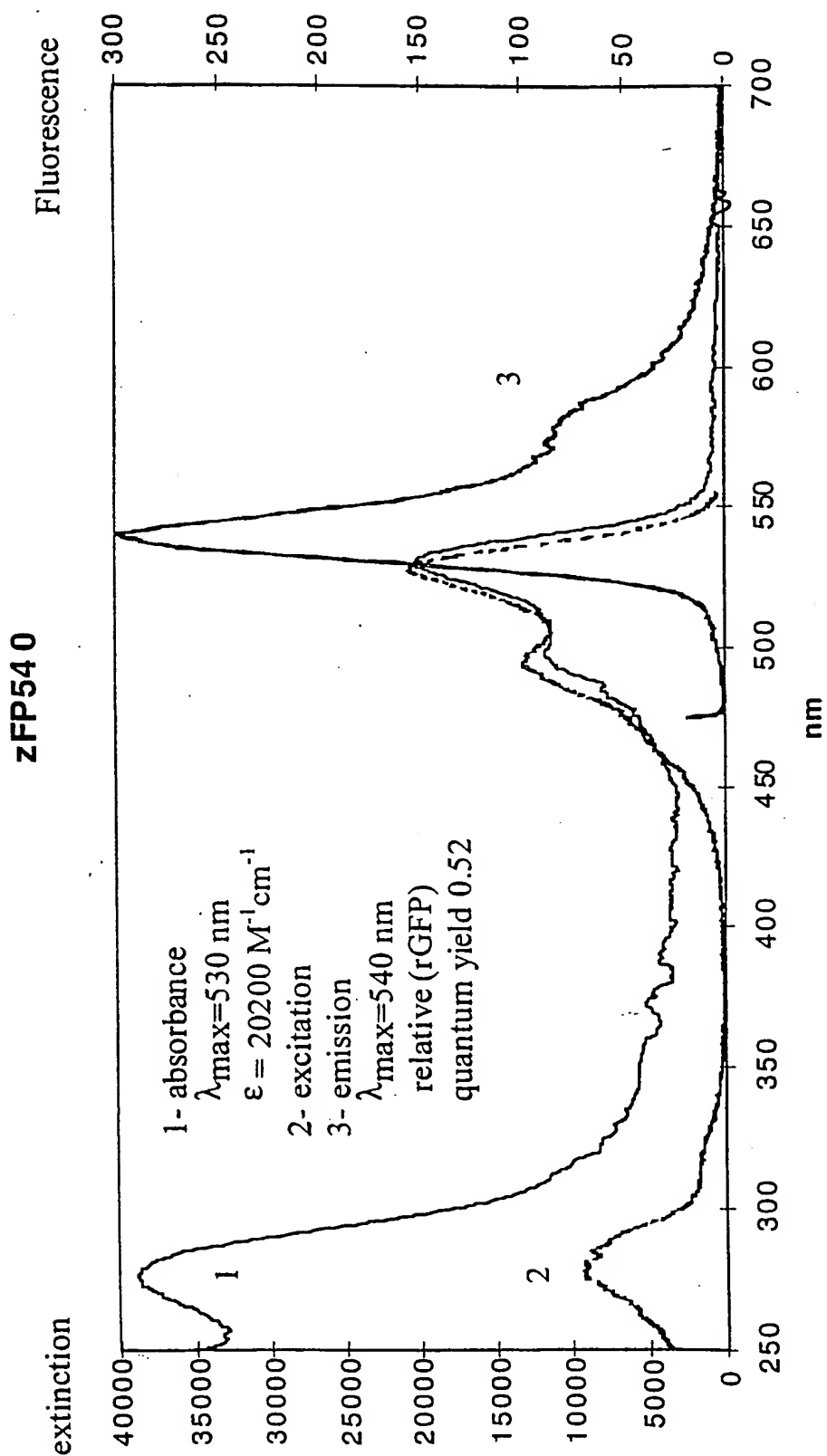
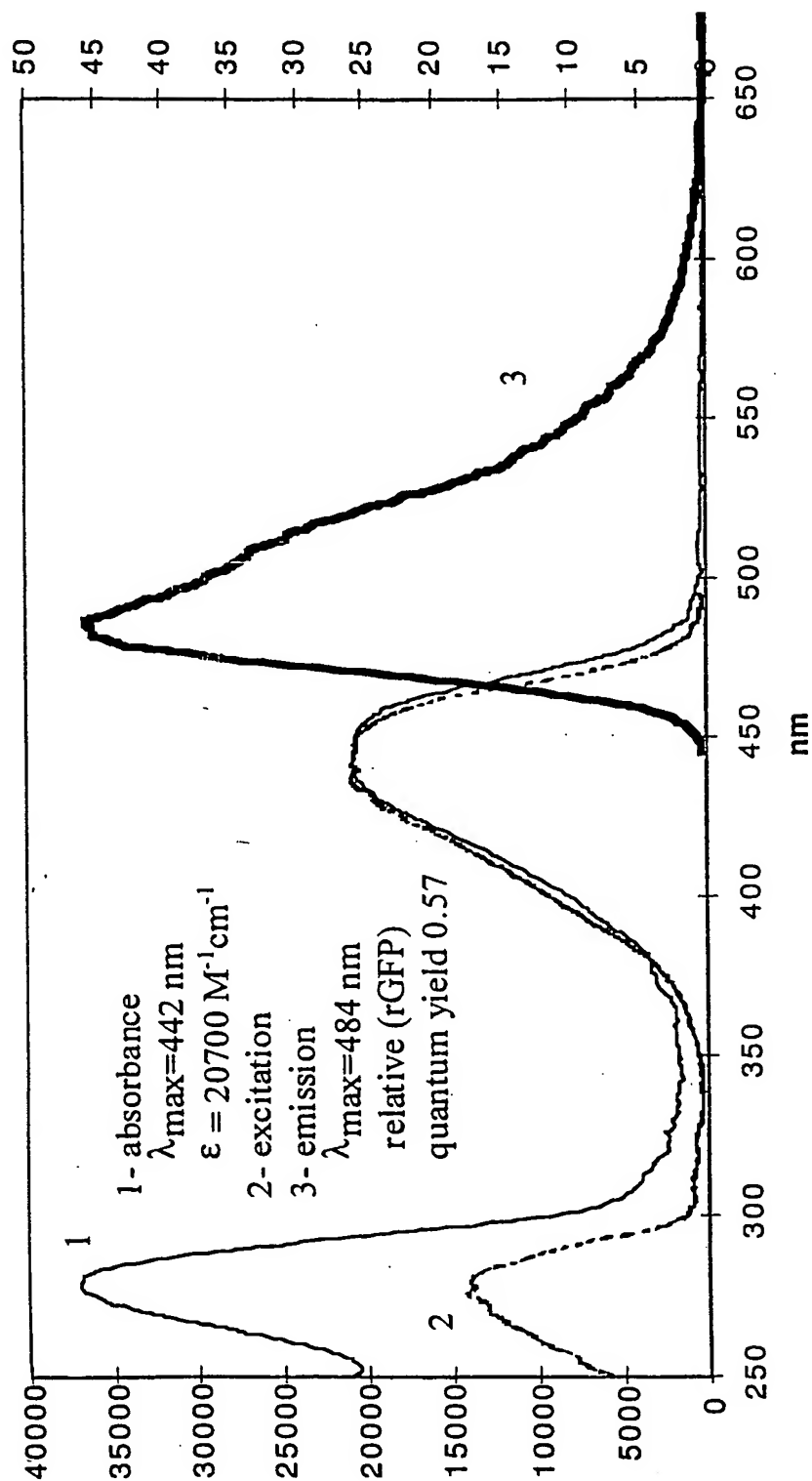


FIG. 6

**dsFP484****FIG. 7**



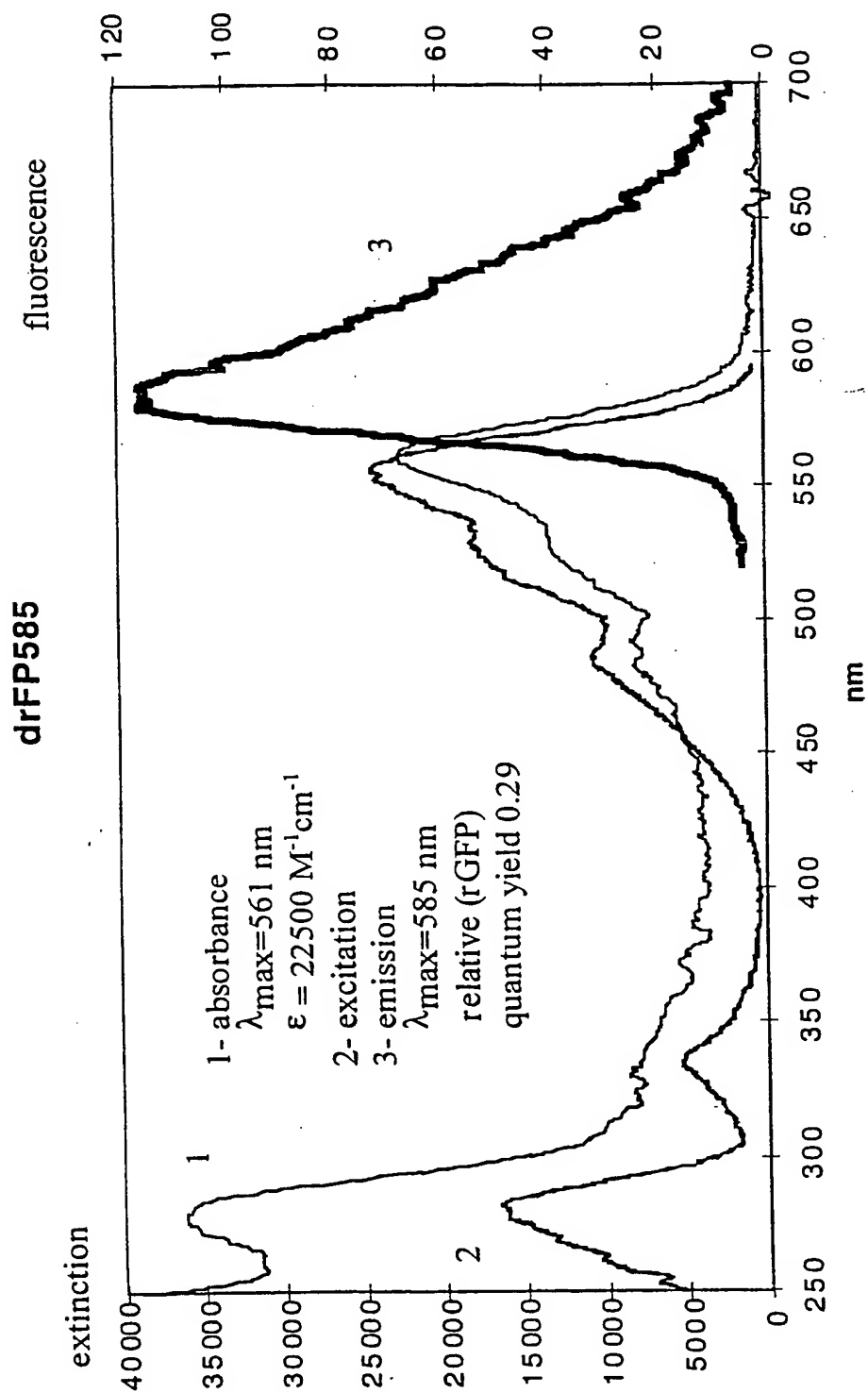


FIG. 8

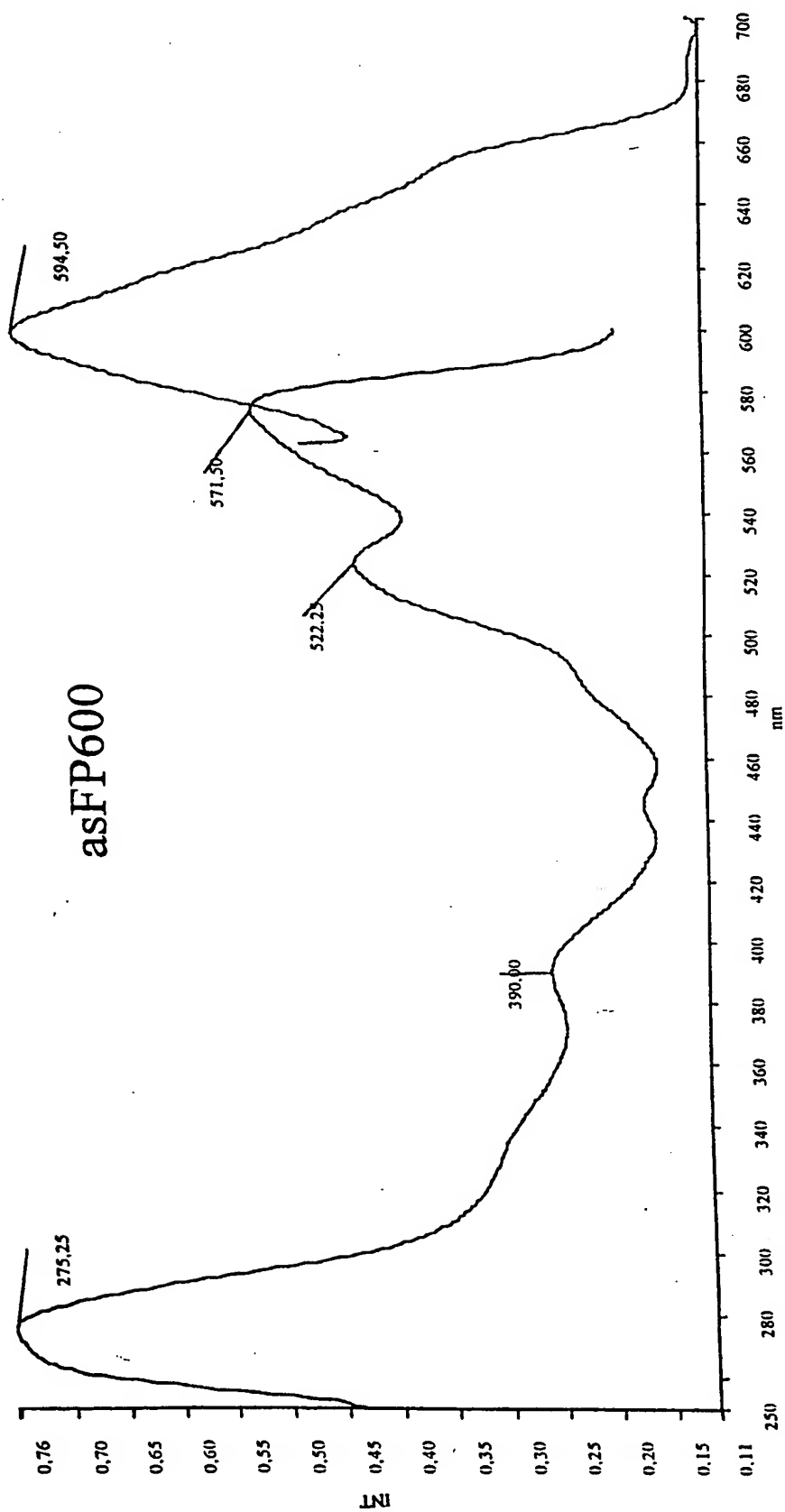


FIG. 9

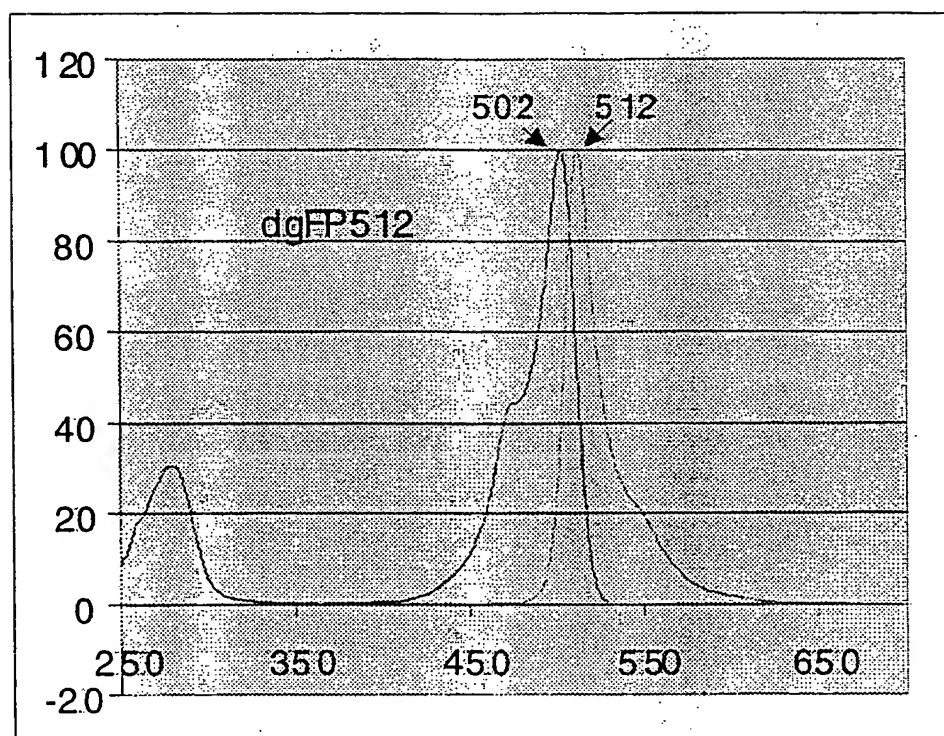


Fig. 10

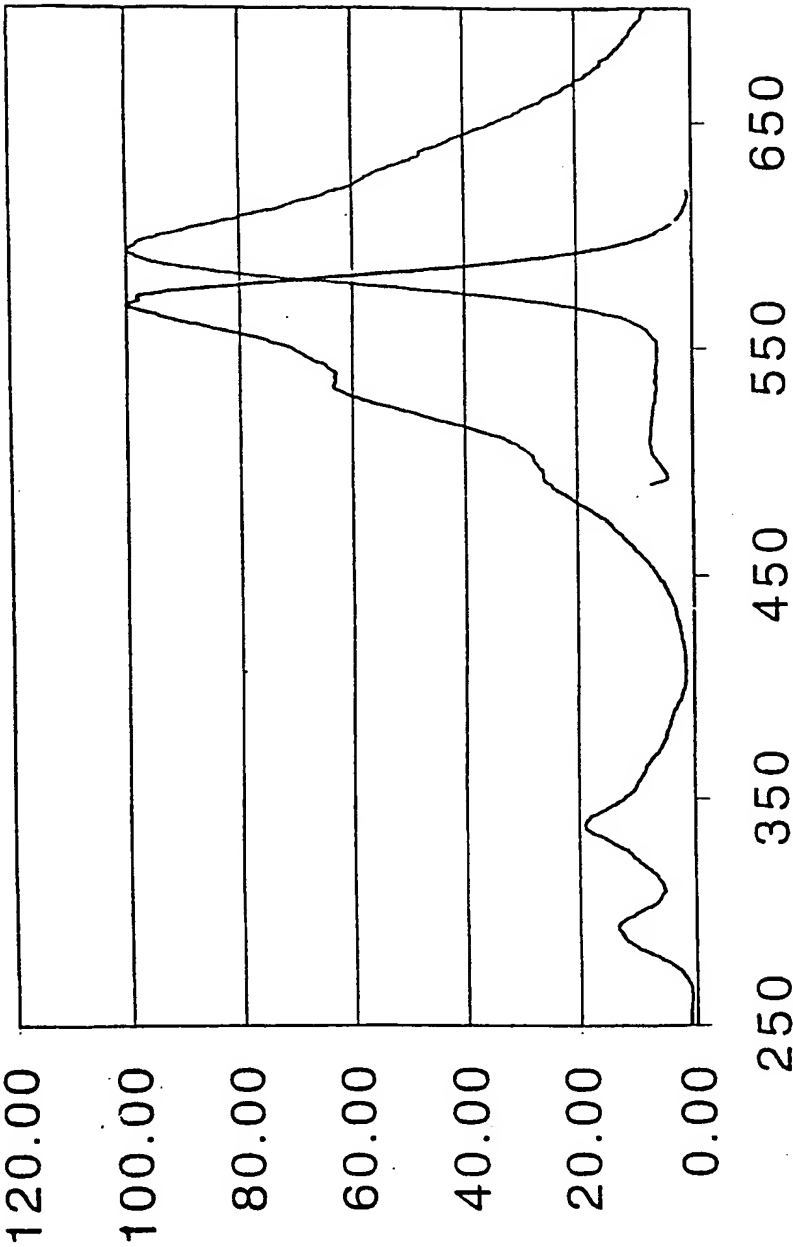


FIG. 11

## SEQUENCE LISTING

<110> Lukyanov, Sergey A.  
 Labas, Yulii A.  
 Matz, Mikhail V.  
 5 Fradkov, Arcady F.  
 <120> Fluorescent proteins from non-bioluminescent  
 species of Class Anthozoa, genes encoding such  
 proteins and uses thereof  
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 10 <141> 1999-12-10  
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 5  
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21

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19

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*Discosoma striata*

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19

5 &lt;210&gt; 29

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17

15

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20 &lt;220&gt;

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&lt;400&gt; 30

25 cctctatctt catttcctgc

20

&lt;210&gt; 31

&lt;211&gt; 20

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&lt;221&gt; primer\_bind

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of nFPs from *Clavularia sp.*

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	Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys		
35		35	40 45
	Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu		
		50	55 60

	Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro		
	65	70	75
	Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu		
	80	85	90
5	Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn		
	95	100	105
	Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val		
	110	115	120
	Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn		
10	125	130	135
	Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val		
	140	145	150
	Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe		
	155	160	165
15	Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp		
	170	175	180
	His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu		
	185	190	195
	Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp		
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	Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr		
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	230	235	
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	Gly Glu Gly Asn Gly Lys Pro Tyr Glu Gly Thr Gln Thr Ser Thr		
	35	40	45

Phe Lys Val Thr Met Ala Asn Gly Gly Pro Leu Ala Phe Ser Phe  
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 Asp Ile Leu Ser Thr Val Phe Lys Tyr Gly Asn Arg Cys Phe Thr  
 65 70 75  
 5 Ala Tyr Pro Thr Ser Met Pro Asp Tyr Phe Lys Gln Ala Phe Pro  
 80 85 90  
 Asp Gly Met Ser Tyr Glu Arg Thr Phe Thr Tyr Glu Asp Gly Gly  
 95 100 105  
 Val Ala Thr Ala Ser Trp Glu Ile Ser Leu Lys Gly Asn Cys Phe  
 10 110 115 120  
 Glu His Lys Ser Thr Phe His Gly Val Asn Phe Pro Ala Asp Gly  
 125 130 135  
 Pro Val Met Ala Lys Lys Thr Thr Gly Trp Asp Pro Ser Phe Glu  
 140 145 150  
 15 Lys Met Thr Val Cys Asp Gly Ile Leu Lys Gly Asp Val Thr Ala  
 155 160 165  
 Phe Leu Met Leu Gln Gly Gly Gly Asn Tyr Arg Cys Gln Phe His  
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 Thr Ser Tyr Lys Thr Lys Lys Pro Val Thr Met Pro Pro Asn His  
 185 190 195  
 20 Val Val Glu His Arg Ile Ala Arg Thr Asp Leu Asp Lys Gly Gly  
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 25 Val Val Pro Phe

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	Asn	Gly	His	Ala	Phe	Val	Ile	Glu	Gly	Glu	Gly	Glu	Gly	Lys	Pro
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	Tyr	Asp	Gly	Thr	His	Thr	Leu	Asn	Leu	Glu	Val	Lys	Glu	Gly	Ala
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		170		175		180									
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		185		190		195									
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25		200		205		210									
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		230		235		240									
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	Glu	Gly	Ile	Gly	Tyr	Pro	Phe	Lys	Gly	Lys	Gln	Ala	Ile	Asn	Leu	
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	Cys	Val	Val	Glu	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Glu	Asp	Ile	Leu	
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	Ser	Ala	Ala	Phe	Asn	Tyr	Gly	Asn	Arg	Val	Phe	Thr	Glu	Tyr	Pro	
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	Thr	Trp	Asp	Arg	Ser	Phe	Leu	Phe	Glu	Asp	Gly	Ala	Val	Cys	Ile	
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	Cys	Asn	Ala	Asp	Ile	Thr	Val	Ser	Val	Glu	Glu	Asn	Cys	Met	Tyr	
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	His	Glu	Ser	Lys	Phe	Tyr	Gly	Val	Asn	Phe	Pro	Ala	Asp	Gly	Pro	
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	Val	Met	Lys	Lys	Met	Thr	Asp	Asn	Trp	Glu	Pro	Ser	Cys	Glu	Lys	
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25	Ile	Ile	Pro	Val	Pro	Lys	Gln	Gly	Ile	Leu	Lys	Gly	Asp	Val	Ser	
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	Met	Tyr	Leu	Leu	Leu	Lys	Asp	Gly	Gly	Arg	Leu	Arg	Cys	Gln	Phe	
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	Asp	Thr	Val	Tyr	Lys	Ala	Lys	Ser	Val	Pro	Arg	Lys	Met	Pro	Asp	
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	Trp	His	Phe	Ile	Gln	His	Lys	Leu	Thr	Arg	Glu	Asp	Arg	Ser	Asp	
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	Ala	Lys	Asn	Gln	Lys	Trp	His	Leu	Thr	Glu	His	Ala	Ile	Ala	Ser	
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 Gly Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Thr Ile Asn  
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 15 Leu Cys Val Ile Glu Gly Gly Pro Leu Pro Phe Ser Glu Asp Ile  
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 Leu Ser Ala Gly Phe Lys Tyr Gly Asp Arg Ile Phe Thr Glu Tyr  
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 Pro Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly  
 20                   80                   85                   90  
 Tyr Thr Trp Gly Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile  
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 Cys Asn Val Asp Ile Thr Val Ser Val Lys Glu Asn Cys Ile Tyr  
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 25 His Lys Ser Ile Phe Asn Gly Met Asn Phe Pro Ala Asp Gly Pro  
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 Val Met Lys Lys Met Thr Thr Asn Trp Glu Ala Ser Cys Glu Lys  
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 Ile Met Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser  
 30                   155                   160                   165  
 Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Tyr Arg Cys Gln Phe  
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 Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Ser Lys Met Pro Glu  
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 35 Trp His Phe Ile Gln His Lys Leu Leu Arg Glu Asp Arg Ser Asp  
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Pro Ser Ala Leu Ala

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 Pro Asp Asn Ile His Asp Tyr Leu Lys Leu Ser Phe Pro Glu Gly  
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 Tyr Thr Trp Glu Arg Ser Met His Phe Glu Asp Gly Gly Leu Cys  
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 Cys Ile Thr Asn Asp Ile Ser Leu Thr Gly Asn Cys Phe Tyr Tyr  
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 Asp Ile Lys Phe Thr Gly Leu Asn Phe Pro Pro Asn Gly Pro Val  
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 30   Val Gln Lys Lys Thr Thr Gly Trp Glu Pro Ser Thr Glu Arg Leu  
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 Tyr Pro Arg Asp Gly Val Leu Ile Gly Asp Ile His His Ala Leu  
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 Thr Val Glu Gly Gly Gly His Tyr Ala Cys Asp Ile Lys Thr Val  
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 Tyr Arg Ala Lys Lys Ala Ala Leu Lys Met Pro Gly Tyr His Tyr  
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Val Asp Thr Lys Leu Val Ile Trp Asn Asn Asp Lys Glu Phe Met  
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 20 35 40 45  
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 50 55 60  
 Leu Ser Pro Gln Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His  
 65 70 75  
 25 Pro Ala Asp Ile Pro Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly  
 80 85 90  
 Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val  
 95 100 105  
 Thr Val Thr Gln Asp Ser Ser Leu Gln Asp Gly Cys Phe Ile Tyr  
 30 110 115 120  
 Lys Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val  
 125 130 135  
 Met Gln Lys Lys Thr Met Gly Trp Glu Ala Ser Thr Glu Arg Leu  
 140 145 150  
 35 Tyr Pro Arg Asp Gly Val Leu Lys Gly Glu Ile His Lys Ala Leu  
 155 160 165  
 Lys Leu Lys Asp Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile  
 170 175 180

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Tyr Met Ala Lys Lys Pro Val Gln Leu Pro Gly Tyr Tyr Tyr Val
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Asp Ser Lys Leu Asp Ile Thr Ser His Asn Glu Asp Tyr Thr Ile
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5 Val Glu Gln Tyr Glu Arg Thr Glu Gly Arg His His Leu Phe Leu
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Glu Gly Asn Pro Phe Glu Gly Thr Gln Glu Met Lys Ile Glu Val
20          35                      40                      45
Ile Glu Gly Gly Pro Leu Pro Phe Ala Phe His Ile Leu Ser Thr
          50                      55                      60
Ser Cys Met Tyr Gly Ser Lys Thr Phe Ile Lys Tyr Val Ser Gly
          65                      70                      75
25 Ile Pro Asp Tyr Phe Lys Gln Ser Phe Pro Glu Gly Phe Thr Trp
          80                      85                      90
Glu Arg Thr Thr Thr Tyr Glu Asp Gly Gly Phe Leu Thr Ala His
          95                      100                     105
Gln Asp Thr Ser Leu Asp Gly Asp Cys Leu Val Tyr Lys Val Lys
30          110                     115                     120
Ile Leu Gly Asn Asn Phe Pro Ala Asp Gly Pro Val Met Gln Asn
          125                     130                     135
Lys Ala Gly Arg Trp Glu Pro Ala Thr Glu Ile Val Tyr Glu Val
          140                     145                     150
35 Asp Gly Val Leu Arg Gly Gln Ser Leu Met Ala Leu Lys Cys Pro
          155                     160                     165
Gly Gly Arg His Leu Thr Cys His Leu His Thr Thr Tyr Arg Ser
          170                     175                     180

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Lys Lys Pro Ala Ser Ala Leu Lys Met Pro Gly Phe His Phe Glu  
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 Asp His Arg Ile Glu Ile Met Glu Glu Val Glu Lys Gly Lys Cys  
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 Lys Gly Gly Pro Leu Pro Phe Ser Tyr Asp Ile Leu Thr Thr Met  
 50 55 60  
 25 Phe Gln Tyr Gly Asn Arg Ala Phe Val Asn Tyr Pro Glu Asp Ile  
 65 70 75  
 Pro Asp Ile Phe Lys Gln Thr Cys Ser Gly Pro Asn Gly Gly Tyr  
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 Ser Trp Gln Arg Thr Met Thr Tyr Glu Asp Gly Gly Val Cys Thr  
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 Ala Thr Ser Asn Ile Ser Val Val Gly Asp Thr Phe Asn Tyr Asp  
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Ile His Phe Met Gly Ala Asn Phe Pro Leu Asp Gly Pro Val Met  
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 5 Glu Arg Asp Gly Met Leu Arg Gly Asp Ile Ala Met Ser Leu Leu  
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 Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly His Cys Ser Val Lys  
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	Thr Val Ser Gln Asp Ser Ser Leu Lys Asp Gly Cys Phe Ile Tyr		
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15	Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala Leu		
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	Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile		
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	Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr		
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25	Ile Lys Pro Leu Gln		
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/29405

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : C12Q 1/68; C07K 14/435

US CL : 435/6, 69.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 968; 530/350; 424/9.6, 436/172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
***	The sequence diskette submitted with the description was defective; thus the references listed below were obtained solely by a WORD search, and not by a search of the SEQ ID NOs.	***
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, entire document.	1-10
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document.	3-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

18 FEBRUARY 2000

Date of mailing of the international search report

02 MAR 2000

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